Poly(*n*-butyl methacrylate) with Primary Amine End Groups for Supporting Cell Adhesion and Proliferation of Renal Epithelial Cells

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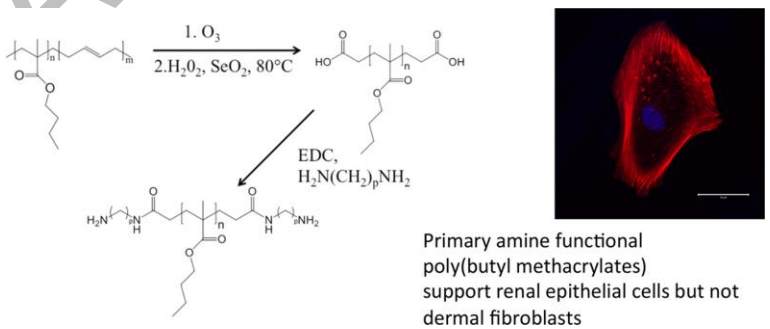
Abstract

Polymer coatings that support epithelial cell culture have been developed. Ozonolysis and subsequent work up of poly(butyl methacrylate-co-butadiene) copolymers is used to form oligomers with carboxylic acid end groups, which are then further reacted with diamines to provide poly(butyl methacrylate)s with primary amine end groups. The polymers are cast as films and used as cell culture substrates for human dermal fibroblasts and human renal epithelial cells. Fibroblast and epithelial cells adhere and proliferate on acid functional materials but on amine functional films epithelial cells show greater viability than fibroblasts.

Graphical Abstract



KEYWORDS: biocoatings, amines, epithelial cells, fibroblast cells

INTRODUCTION

Surface coatings that can modify the cell adhesive properties of medical devices are important in both regenerative medicine and cell therapy technologies. However, the currently available approaches tend not to select for particular cells. For example, peptide functional materials (mainly containing the RGD peptide) bind to integrins and induce spreading and adhesion(1) on otherwise non-adhesive surfaces but there are few examples of the selective use of peptides or other groups that support the adhesion of particular cell types in preference to others. Several studies have shown that adding either positive or negative surface charge(2-5) can influence cell adhesion and a number of these reports indicated that positive charge, often added via modification with quaternary ammonium functionality, had a preferential effect on the adhesion of fibroblasts(6-9) but other reports showed that in some systems surface charge, derived from quaternary amine functionality, had little effect on cell adhesion.[10] Physical features of materials (such as surface topography, swelling and mechanical properties) also influence cell adhesion.(11) However, although optimisation for particular cells maybe be possible in the future, there is little evidence currently that controlling these physical materials parameters can provide substrates that are selective for particular cell types. On the other hand we recently showed that functionalisation of hydrogels with primary alkyl amines provided substrates that supported corneal epithelial cells but not fibroblasts.(12) These hydrogels are excellent bulk materials for medical devices but producing coatings from them is not

facilitated by the requirement to polymerize and cross-link unsaturated monomers: usually by radical polymerisation. Therefore, here we have designed a coating system based on the use of poly(butyl methacrylate) with amine end groups (PBMA-NH_2) and we report comparative studies of cell adhesion with human dermal fibroblasts (HDF) and human renal epithelial cells (REpC).

EXPERIMENTAL

Materials

Butadiene (99+%, Aldrich), potassium persulphate (99+%, Aldrich), potassium hydrogen phosphate (98+%, Aldrich), Dowfax 2A1 surfactant solution (alkyldiphenyloxide isulphonate, Dow chemical company, USA), sodium chloride (Fisher, Lab reagent grade), methanol (Fisher, HPLC grade) and beta-cyclodextrin hydrate (Alfa Aesar) were used as received. Butyl methacrylate (99%, Aldrich) was purified before use to remove any inhibitor present. It was twice washed with equal portions of aqueous 2 wt% sodium hydroxide solution, followed by two equal portions of distilled water. This washed butyl methacrylate was then distilled under reduced pressure, prior to use and stored at –4 ˚C. Deionised water was used throughout. 1,2 diaminoethane (99%, Aldrich), 1,3-diaminopropane (99%, Aldrich) 1,4-diaminobutane (99%, Aldrich), 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (Alfa Aesar) were used as received. Hydrogen peroxide (35% v/v, Alfa Aesar), selenium dioxide (98%, Aldrich), toluene (Fisher) and Amberlite IRA 400(Cl) (Aldrich) were used as received. Ozone was generated by passing oxygen through an electrical discharge generator (Type BA, Wallace & Tiernan, UK) at a rate of 1.74 g $hour^{-1}$.

Emulsion Copolymerisation Of Butyl Methacrylate And Butadiene

Poly(butyl methacrylate-co-butadiene) (poly(BMA-co-BD)) was prepared by semi-continuous emulsion polymerisation. Polymerisations were performed in a 1L jacketed glass reactor (Radleys, UK) equipped with an overhead mechanical glass stirrer, a nitrogen inlet, a reflux condenser, a thermocouple probe and inlets for gaseous and liquid monomer addition. Liquid monomers were added using a peristaltic pump (Watson Marlow 505S) and gaseous butadiene was added using an infusion pump (Precidor Type 5003, Infors HT) fitted with 2 gas syringes operating in opposition.[1] β-cyclodextrin (3.6g, 0.00317moles), Dowfax 2A1 (3.6g) and potassium hydrogen phosphate (0.4g) were added to deionized water (450 cm^3) and stirred until dissolved. The solution was added to the reactor and purged with nitrogen for one hour and then heated to 80˚C. At the same time, potassium persulphaate was dissolved in dissolved water (50 cm^3) and butyl methacrylate was added to a two-necked round bottom flask. Both were purged with nitrogen for one hour. Once the contents of the reactor had reached 80 ˚C, the initiator solution was injected into the reactor and the monomer feeds of BD and BMA were started. BMA (100 cm^3, 0.63 moles)) were added at a rate 0.1 cm^3 $min.^{-1}$ and butadiene (1 mole) was added at 24 cm^3 $min.^{-1}$ The reaction was carried out at 80 ˚C for 16 hours.

When both monomers had been consumed, the resulting white latex was removed from the reactor and stored at room temperature. For characterisation the solid polymers were retrieved from the latex by coagulation using aqueous sodium chloride. The white solid

was filtered and washed thoroughly with water and then methanol before being dried in a vacuum oven at 50 ˚C.

Synthesis Of Poly(Butyl Methacrylate) With Carboxylic Or Amine End Groups (PBMA-COOH)

The poly(BMA-co-BD) latex (200 cm^3) was added to a 1L 3-neck round bottom flask, equipped with a magnetic stirrer and dropping funnel. An equal volume of deionised water (200ml) was added with stirring. Toluene (50ml) was added dropwise to the diluted latex over 4 hours at room temperature. The swollen latex was then stirred for a further 24 hours. After this time a reflux condenser was added to the flask and it was placed in an ice bath. A glass inlet was used to introduce ozone at a rate of 1.74 g $hour^{-1}$ for 6-8 hours, with constant stirring of the latex throughout. The vessel was then purged with a nitrogen inlet for an hour to remove any residual ozone. Selenium dioxide (4 g) and hydrogen peroxide solution (35% v/v) (100 ml) were then added to the colloidally stable latex with stirring. This was then heated to 80 ^oC for 24 hours. To remove the surfactant, Amberlite IRA 400(Cl) (20g) was mixed with the latex (100 cm^3) with gentle agitation for about 5 hours then the resin was replaced with fresh Amberlite IRA 400(Cl) (20g) and agitated gently overnight. The latex was then removed and dialysised (Visking tubing) against deionised water with twice daily water changes over a period of one week. The PBMA-COOH latexes were then removed from the dialysis tubing and any toluene present was removed under reduced pressure. For analysis, the water was removed azeotropically, by addition of ethanol, under reduced pressure.

The PBMA-COOH latex (75ml) was added to a 3-neck round bottom flask, equipped with a condenser and magnetic stirrer bar. Dowfax 2A1 was then added to the flask and the vessel was cooled in an ice bath with vigorous stirring. 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) in water (45 cm^3) was added slowly to the vessel. After completion of the addition, the latexes maintained colloidal stability. The appropriate diamine was then added to the stirring latex slowly over a period of at least one hour. A substantial exotherm was observed at this stage. After one hour the reaction was left to warm to room temperature and continued to stir for 24 hours. The stable latexes were removed and dialysised (Visking tubing) against distilled water for 2 weeks, with daily changes of water.

Pbma-Cooh

^{1}H NMR (400MHz, THF): δ/ppm 0.98 -$(CH_2)_3C\mathbf{H}_3$), 1.08 (-C$\mathbf{H}_3$), 1.46 (-C$\mathbf{H}_2CH_3$), 1.65 (C$\mathbf{H}_2CH_2O$-), 1.86 (-C$\mathbf{H}_2C(CH_3)(C(=O)O(CH_2)_3CH_3$), 3.95 (-C$\mathbf{H}_2OC=OC(CH_3)$)

Pbma-Nh$_2$

^{1}H NMR (400 MHz, THF): δ/ppm 0.98 -$(CH_2)_3C\mathbf{H}_3$), 1.08 (-C$\mathbf{H}_3$), 1.325 (N$\mathbf{H}_2$), 1.46 (-C$\mathbf{H}_2CH_3$), 1.65 (-C$\mathbf{H}_2CH_2O$-), 1.86 (-C$\mathbf{H}_2C(CH_3)(C(=O)O(CH_2)_3CH_3$-), 2.53 (-C$\mathbf{H}_2$ NH_2), 3.95 (-C$\mathbf{H}_2OC=OC(CH_3)$), 5.54 (C=ON$\mathbf{H}CH_2$-)

The quantities used for each reaction are shown in Table 1.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) was carried out using 3 x 30 cm Styragel 5mm mixed gel B columns (Agilent). The eluent was THF (GPC grade, Fisher) at a flow rate of 1.0 cm^3 min^{-1}. The calibration was carried out using polystyrene standards. RI detection was employed using an ERC-7512 detector, obtained from ERMA inc. The detectors were attached to a PC running Cirrus software. The samples were injected automatically with a Gilson autosampler. The use of GPC in this way, with polystyrene standards, provides a good estimate of the distribution of molar masses if the Mark Houwink constants for the PBMA polymers and polystyrene are similar. However, within this limitation the molar mass averages are presented relative to the standards and should not be regarded as absolute values.

Culture Of Fibroblast And Epithelial Cells

Cell cultures were performed under sterile conditions in a class II laminar flow hood, using 70% alcohol and Virkon® as disinfectants.

Hrepcs Culture

Materials

Renal epithelial cell growth medium 2 (PromoCell), Cryopreserved HREpC (PromoCell, Germany).

Method

HREpCs were cultured in renal epithelial cell growth medium 2 (PromoCell, ready-to-use) supplemented with fetal calf serum (0.05 ml ml^{-1}) and Epidermal growth factor (10

ng ml^{-1}) Insulin (5 ug ml^{-1}), Epinephrine (0.5 ug ml^{-1}), Hydrocortisone (36 ng ml^{-1}), Transferrin, holo (5 ug ml^{-1}) and Triiodo-L-thyronine (4 pg ml^{-1}). 10 ml of complete medium was placed into T75 flask in the incubator in a humidified atmosphere of 5% CO_2, at 37 ^{0}C for 30 minutes. Cryopreserved HREpCs were placed in a 37 ^{0}C water bath for 90 s with constant agitation. The cells were pipetted up and down and then quickly transferred to the pre-warmed flask. The cells were incubated for a minimum of 16 hours before changing the medium. Subsequent media changes were performed every 2-3 days until the cells were confluent.

Hdfs Culture

Materials

Primary normal dermal fibroblasts cells (ATCC), *Dulbecco's modified eagle medium with HEPES buffer, high glucose, L-Gultamine and Pyruvate (life technologies), Penicillin/streptomycin (100X, Invitrogen), Fetal bovine serum (Sigma).*

Method

60 ml of DMEM was removed from the 500 ml bottle, and replaced with 50 ml of FBS and 10 ml penicillin/streptomycin. 10 ml of complete medium was placed into T75 cell culture flask, and warmed in the incubator for 30 minutes. Cryopreserved HDF cells were placed in a 37^{0}C water bath for 90 sec with constant agitation. The cells were pipetted up and down and then quickly transferred to the pre-warmed flask. The cells were placed in an incubator at 37^{0}C, 5% CO_2 for 24 hours before the media was changed. Subsequent media changes were performed every 2-3 days.

Culture Of Cells In Direct Contact With Oligomers

Functionalized polymers (PBMA-COOH or PBMA-NH_2) were dissolved in 2-propanol at a concentration of 5 mg ml^{-1}. The polymer solutions were well agitated and sonicated for 30 seconds. To produce coated coverslips, a polymer solution (100 μl) was pipetted onto a sterile glass coverslip. An IR lamp was used to remove the solvent. At this point, each coated coverslip was placed into a well of a six well plate and was then washed with sterile PBS to remove residual solvent.

HDFs or HREpCs were treated with trypsin/EDTA and were seeded directly onto the films at a density 1 x 10^4 cells/well. 2 ml of media was added to each well and the cells were incubated for 24 hours at 37 ºC and 5% CO_2. After 24 hours phase contrast imaging and a full media change was performed. After 72 hours cells were assayed and fixed for staining.

Phalloidin-TRITC And DAPI Staining

The medium was removed and the cells were washed twice with PBS. The cells were then fixed with 3.7% formaldehyde in PBS for 30 minutes at room temperature. The formaldehyde was then removed and the cells were washed with PBS twice. Triton X100 solution (1 wt% in PBS) was added to the cells and this was placed in a fridge for 5 minutes. The Triton solution was then removed and the cells were washed with PBS three times. Phalloidin-TRITC (200 μl) and DAPI (300 μl) solutions were added to each

sample, which was then incubated for an hour at 37 ^{0}C and 5% CO_2. The medium of phalloidin and DAPI were then removed and the cells were washed X3 with PBS.

Imaging

Following cell fixation and staining, cells were placed on a Zeiss LSM710 confocal laser scanning microscope (CLSM), with excitation wavelengths of 405 and 564 nm corresponding to DAPI and TRITC respectively.

RESULTS AND DISCUSSION

Synthesis Of Carboxylic Acid And Primary Amine Telechelic Polymers

The synthesis of a set of PBMA-NH_2s was carried out as shown in Figure 1. An aqueous latex of poly(butyl methacrylate) with carboxylic acid end groups (PBMA-COOH) was prepared via ozonolysis of a poly(butyl methacrylate-co-butadiene) latex as previously described.(13) The process produced polymer that was much more disperse than our previously reported material. This broader molar mass distribution was obtained by changing the feed rates of the monomers and is derived from variation in the instantaneous fraction of butadiene incorporated during the emulsion copolymerisation of butyl methacrylate and butadiene.

The PBMA-COOH latex was then reacted with excesses of a range of diamine-functional compounds: 1,2-ethandiamine (1,2-ED); 1,3-propandiamine (1,3-PD), and 1,4-butandiamine (1,4-BD). The reaction was mediated with (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) (EDC), to produce materials with primary amine

end groups (PBMA-1,2NH_2, PBMA-1,3NH_2, PBMA-1,4NH_2). The amidation reactions were performed in a two-step process whereby the oligomer latex and EDC were first charged into the reaction vessel, followed by the slow addition of diamine. The initial reaction of EDC with the carboxylic acid groups was expected predominantly to occur at the aqueous/polymer interface with EDC mainly present in the aqueous phase and the carboxylic acid groups of the PBMA-COOH residing at the interface. Size exclusion chromatographic (SEC) analysis of the functionalized polymers is summarized in Figure 2. Amination with excess amine produced an increase in the molar mass averages as the chains were extended. However, the use of excess amine ensured that the majority of the chains ends were functionalized with primary amine groups. Figure 2 shows also the SEC derived molar mass distributions of the amine and carboxylic acid functional oligomers. The uneven distribution of the butadiene comonomer produced a PBMA-COOH with a broad molar mass distribution, which became narrower and shifted to higher average molar masses after reaction with excess diamine.

The narrowing of the molar mass distribution is a consequence of the interfacial polymerisation process. In this system lower molar mass oligomers would be present at proportionally higher concentration at the aqueous/polymer interface than higher molar mass PBMA, which contain a smaller fraction of carboxylic acid groups relative to the repeat units. We expect the chain extension process to be very similar to conventional interfacial polymerisation so that low molar mass oligomers located at the interface will react rapidly, whereas higher molar mass PBMA containing carboxylic acid groups buried within the hydrophobic particles should react more slowly because they must

diffuse to the interface. Polymerisations following this model would result in low molar mass oligomers rapidly increasing in size with higher molar mass polymers increasing in size much more slowly, resulting in a narrowing of the molar mass distribution.

Contact angles were obtained for films formed from the acidic oligomer (54.4º ± 1.2) and the 1,2-diamine functionalized oligomer (4.75º ± 0.17). However, values for the other materials were not easily obtainable as the droplets quickly dispersed on the surface of the material. These results confirmed the successful modification of the PBMA.

Cell Contact Studies

Human Dermal Fibroblasts (Hdfs)

PBMA-COOH and the PBMA-NH_2s were dissolved in *iso*propanol and cast onto sterile glass coverslips. Then coherent coatings were produced by forced drying with an infra-red lamp. HDF cells were seeded on the coated coverslips and their viability was assessed using the Alamar blue assay. Empty wells (tissue culture plastic, TCP) were used as the controls. The data are summarized in Figure 3A from two separate experiments. In both experiments the PBMA-COOH gave lower values than the TCP control ($p < 0.01$). Also, in both experiments the PBMA-NH_2s performed worse as substrates for supporting cells compared to the control ($p < 0.01$, not shown). Importantly, all of the average values were lower for the PBMA-NH_2 coatings than the PBMA-COOH ($p < 0.05$).

Optical microscopy was then used to investigate the morphology of the adhered cells (Figure 3 B-F). In all experiments the cells were incubated on coated coverslips. These images showed that the coatings with acid functionality were covered with the largest amount of cells and their morphology and density was similar on TCP and PBMA-COOH. Fibroblast morphology on the aminated oligomers was abnormal and the cells were spindly and spaced sparsely. Figures 3 G-I show additionally images of the HDFs, with staining of the actin cytoskeleton and nucleus, on the amine functional coatings. The images show a few cells were attached with well-defined actin cytoskeletons but they had a rounded appearance rather than the more familiar extended appearance of HDFs in culture.

Human Renal Epithelial Cells (Hrepc)

In the previous section it was shown how the primary amine functional coatings were not useful substrates for supporting fibroblast cells and this reconfirmed our previous report showing that fibroblast culture could not be supported on alkyl primary amine-functional hydrogels.(12) However, it was also shown, (12, 14) as well as by Lin et al,(15) that primary amine funtionality was useful for supporting epithelial cells.

Therefore, to investigate the utility of theses primary amine-functional coatings as supports for epithelial cells, primary renal epithelial cells were cultured in direct contact with the coatings. The viability assay results are shown in Figure 5A. These data showed that the HREpC responded quite differently to amine-functional coatings compared to the fibroblasts. Whereas the experiments with fibroblast culture showed a clear preference of

these cells for PBMA-COOH coating the HREpCs were supported on all of the PBMA-NH_2 and the PBMA-COOH coatings. Thus, the data indicated that the PBMA-NH_2 were good substrates for the HREpCs but they were poor substrates for the fibroblasts. Figure 4 also shows images of the HREpCs (72 hours) on each of the PBMA-NH_2 coatings. The images show the familiar actin cortex around the edges of these epithelial cells and there was some indications that the PBMA-1,2NH_2 and PBMA-1,3NH_2 were superior substrates when compared to the PBMA-1,4NH_2 coating; semi-qualitative observations indicated that there were larger numbers of well-formed cells on PBMA-1,2NH_2 and PBMA-1,3NH_2.

Our previous work showed that low molar mass (Mn < 2700 g mol^{-1}) PBMA with carboxylic acid end groups (PBMA-COOH) was cytotoxic.(16) However, here we have shown that it is possible to produce PBMA-COOH with a low number average molar mass (Mn = 1400 g mol^{-1}) that is not toxic to either human dermal fibroblasts or human renal epithelial cells provided the low molar mass material is diluted with higher molar mass material: i.e. the molar mass distribution is broadened. Reaction of the PBMA-COOH with an excess of an alkyl diamine produced a series of polymers with alkyl amine end groups. It was shown that coatings formed from PBMA-COOH were good substrates for supporting the culture of HDF and HREpCs. Following on from other previous work involving hydrogel membranes with primary amine functionality[12, 14] the work reported here shows that primary amine-functional materials can be used to support epithelial cells but not fibroblasts. Our early work had provided some indication that co-culture with stromal cells was advantages to supporting human corneal epithelial cells

(HCECs) on amine-functional hydrogels but latter work by use(14) showed that this was not the case and that epithelialisation with HCECs was achieved with monoculture.(12) The data here support those observations and although more work is required to establish the mechanism of action of these systems these aminated oligomers provide a useful coating system to promote epithelialisation on medical devices.

CONCLUSION

Coatings composed of oligo(butyl methacrylate) with primary amine end groups support the culture of human renal epithelial but not human dermal fibroblast cells.

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Table 1. Table of reagents used to functionalize PBMA-COOH to provide PBMA-NH_2.

Diamine	**Amount used/g (mol)**	**EDC/ g**	**DOWFAX2A1 /g**
1,2 diaminoethane	4.651 (0.08)	3.6971	1.210
1,3-diaminopropane	5.841 (0.08)	3.7123	1.210
1,4-diaminobutane	6.812 (0.09)	3.6991	1.210

Figure 1. Synthesis of PBMA-COOH and PBMA-NH_2 coatings

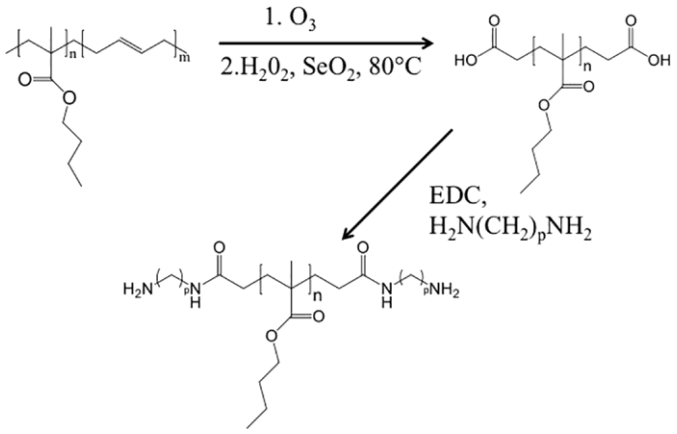

Figure 2. Molar mass averages and distributions of synthesised PBMA-based materials

Oligomer	$\bar{M}_n$/g mol^{-1}	$Đ_M$
P(BMA-co-BD)	16300	2.7
PBMA-COOH	1400	10.1
PBMA-1,2NH$_2$	12250	3.7
PBMA-1,3NH$_2$	11400	4.0
PBMA-1,4NH$_2$	11600	3.9

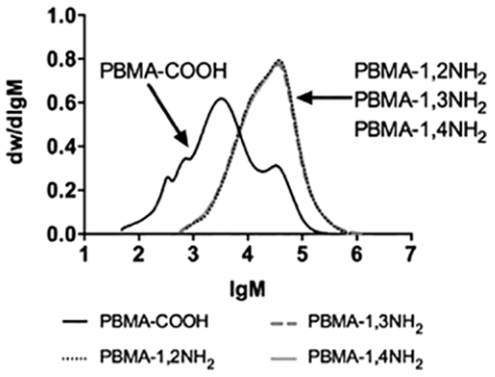

Figure 3. A AlamarBlue® results after incubation of HDFs for 72 hours in direct contact with PBMA-COOH or the PBMA-2NH_2s. Contact experiment repeated on two separate occasions (1 and 2). Error bars are standard error of the mean. Data analysed with one-way analysis of variance and Fisher's LSD *post-hoc* procedure. Significance of pairwise comparisons: $p < 0.05$ *; $p < 0.01$ ** or $p < 0.001$ ***. The data are displayed as the number of viable cells assuming equal response of the cells to the AlamarBlue® procedure. **B-F** images (20x) of fibroblasts on functionalized oligomers and TCP. Cells cultured on oligomers for 72 hours then fixed with 10% formalin and stained with Giemsa's stain: B = PBMA-1,2NH_2; C = -PBMA-1,3NH_2; D = PBMA-1,4NH_2 functional; E = PBMA-COOH; F = TCP. **G-I** Fibroblasts on G = PBMA-1,2NH_2; H = PBMA-1,3NH_2; I = PBMA-1,4NH_2 coatings: stained with phalloidin-tetramethyl rhodamine (for Actin) and DAPI (nucleus).

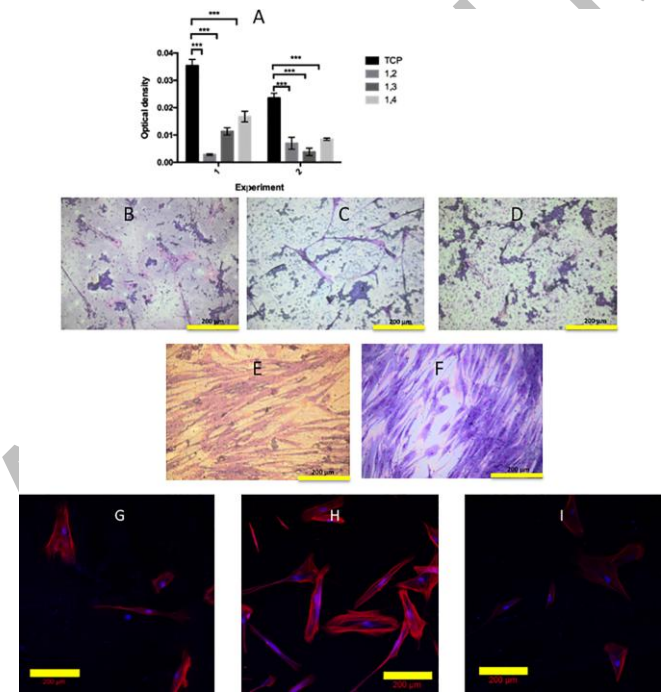

Figure 4. Viability of HREp cells and micrographs of cells on the coatings. A. AlamarBlue® results of 72 hour incubation in direct contact with oligomers. Data analysed with one-way analysis of variance and Fisher's LSD procedure. Significantly different results *** $p < 0.001$. **B, C, D.** Micrographs of HREp on various substrates: red-actin and blue-nuclei. B = PBMA-1,2NH_2; C = -PBMA-1,3NH_2; D = PBMA-1,4NH_2